## AD NUMBER

**ADB248365**

## NEW LIMITATION CHANGE

TO

**Approved for public release, distribution unlimited**

FROM

**Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Oct 98. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Fort Detrick, MD 21702-5012.**

## AUTHORITY

**U.S. Army Medical Research and Materiel Command and Fort Detrick ltr., dtd October 17, 2001.**

AD_____

GRANT NUMBER DAMD17-97-1-7268

TITLE: Apoptosis and Tumor Invasion in Breast Cancer

PRINCIPAL INVESTIGATOR: Martin Tenniswood, Ph.D.

CONTRACTING ORGANIZATION: W. Alton Jones Cell Science Center, Inc.
Lake Placid, New York 12946-1099

REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | August 1998 | Annual (1 Aug 97 - 31 Jul 98) |

**4. TITLE AND SUBTITLE**

Apoptosis and Tumor Invasion in Breast Cancer

**5. FUNDING NUMBERS**

DAMD17-97-1-7268

**6. AUTHOR(S)**

Tenniswood, Martin, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

W. Alton Jones Cell Science Center, Incorporated
Lake Placid, New York 12946-1099

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Distribution authorized to U.S. Government agencies only (proprietary information, Oct 98). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 words)*

We have created a series of breast cancer cell lines, transfected with green fluorescent protein (GFP) that can be use to examine the effects of anti-estrogens on the invasive potential of breast cancer cells that survive anti-estrogen therapy. We have tested 20 of these cell lines to establish that they have retained their sensitivity to tumor necrosis factor-α (TNFα) and to several anti-estrogens (tamoxifen, 4-hydroxytamoxifen and ZM 182,780). Most, but not all of the cell lines induce apoptosis after treatment with anti-estrogens. After 72 h of treatment 60-70% of the transfected cells die by apoptosis, while 40% of the cells survive. During the apoptotic process the cells induce the transcription of several extracellular matrix proteases (particularly cathepsin B and MMP-9) and down regulate the expression of at least one matrix metalloprotease inhibitor (TIMP-1). These data provide support for our hypothesis that while anti-estrogen therapy induces cell death in the majoroty of hormone responsive cells, a same population of cells may survive the treatment but may induce the enzymes required for invasion. If further experimentation demonstrates directly that there is an increase in the invasive potential of the surviving cells it will suggest anti-estrogen therapy for early stage disease or as a chemo-preventive strategy is contra-indicated.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| Breast Cancer | 9 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Limited |

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

MPRT For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

MPRT In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

MPRT In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Mark Ironswood 10/20/98

PI - Signature Date

## TABLE OF CONTENTS

# Annual Report for Grant Number DAMD 17-97-7298

Title: Apoptosis and Tumor Invasion in Breast Cancer

The research outlined in this application is designed to test the hypothesis that the acquisition of the invasive phenotype may result if apoptosis is initiated but endonuclease activation is abrogated.

In the original application there were two specific aims:

**Specific Aim 1**. *To characterize the ability of anti-estrogens to induce apoptosis and an invasive phenotype in a sub-population of cells in vitro.*

**Specific Aim 2.** *To compare thesensitivity of MCF-7$^{ae}_{inv}$ sublines to other agents that induce apoptosis and determine if multiple agents can act synergistically to induce apoptosis.*

Prior to the initiation of the research we were asked to describe how we would pursue this work in vivo as part of a follow up study. At that time we added specific aim 3 to the research program, with the understanding that this specific aim was outside the scope of the two funding period.

**Specific Aim 3:** *To determine whether treatment with anti-estrogens or other apoptosis inducing reagents (such as vitamin D analogs) alters the rate and/or sites of metastasis and whether invasive cells generated by treatment with one reagent are sensitive to treatment with other reagents that induce cell death in vivo.*

While specific aim 3 was clearly not part of the original application, our proposal to use MCF-7 cells transfected with GFP (green fluorescent protein) to monitor invasive cells both *in vitro* and *in vivo* suggested to us that we would be well advised to create these MCF-7$^{GFP}$ sublines before proceeding to examine the effects of anti-estrogens on these breast cancer cell lines to ensure that the transfected cells retained their normal estrogen dependence and their responsiveness to anti-estrogens.

We have therefore modified our original experimental timetable to include an additional preliminary task: the development of estrogen sensitive clonal cell lines expressing enhanced Green Fluorescence Protein (GFP). These cell lines have been developed by transfecting estrogen dependent MCF-7 cell with pEGFP-c1, a plasmid containing the enhanced green fluorescent protein under the control of the constitutive CMV promoter, and an ampicillin selectable marker. These cells were cultured for 7 days, and then sorted on a Becton-Dickinson fluorescence activated cell sorter into 96-well culture plates using the autosort facility of the machine. The cells in individual wells were clonally were expanded to produce a series of high, medium and low GFP-expressing

***[This page contains unpublished data]***

sublines by growth in G418. A total of 20 sublines have been stabilized, characterized and frozen in liquid nitrogen for further use. Phase contract and fluorescence photomicrographs of one of these sublines is shown in Figure 1, demonstrating that virtually all of the cells within the selected subline express GFP at approximately the same level. [ A color version of Fig 1 is provided in appendix 1.]

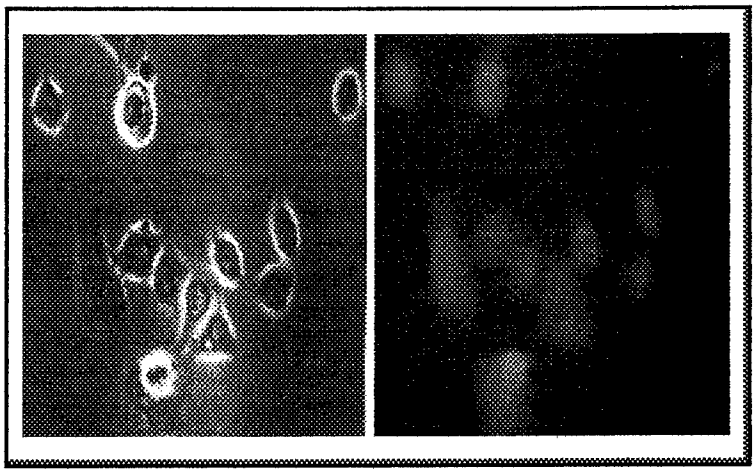


**Figure 1**: Phase contrast (left panel) and fluorescence microscopy (right panel) of MCF-7 cells transfected with a plasmid expressing green fluorescent protein (GFP). MCF-$7^{GFP}$cells were grown for 72 h in serum free defined medium prior to photography.

In our initial experiments we have compared the sensitivity of these sublines to TNF-α with that of the parental MCF-7 wild type cells (MCF-$7^{WT}$). Without exception, the MCF-$7^{GFP}$, retain their sensitivity to TNF-α and demonstrate a very similar dose response to the drug as the parental wild type MCF-7 cells, suggesting that the expression of GFP in these cells does not influence the ability of the transfected cells to undergo apoptosis after the appropriate stimulation (Fig. 2).

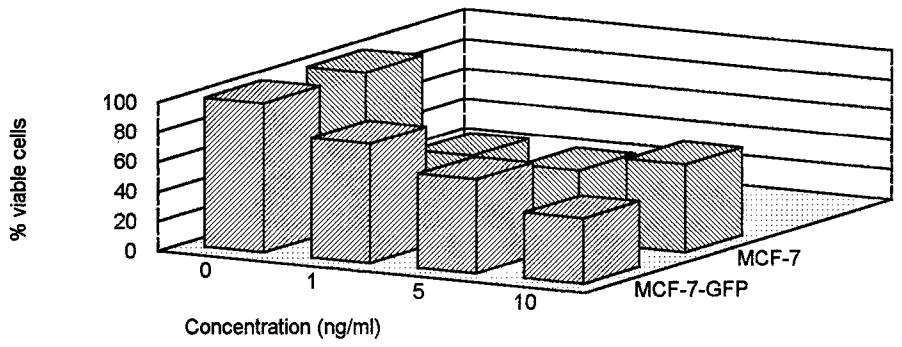



**Figure 2**: Comparison of sensitivity of MCF-$7^{GFP}$ and parental MCF-$7^{WT}$ to increasing doses of TNF- α. The two cell lines were grown for 4 days and then treated with increasing doses of TNF-α. After 72 h the number of surviving cells was estimated using crystal violet, and compared to untreated controls. Results are the mean of three independent experiments.

***[This page contains unpublished data]***

Most, but not all of the transfected sublines have also retained their sensitivity to anti-estrogens and induce cell death (as monitored by DNA fragmentation (TUNEL positivity) and cell viability as monitored by crystal violet or flow cytometry. The relative sensitivity of the subline shown in Fig 1 and 2, to ZM182,780, tamoxifen and 4-hydroxytamoxifen is shown in Fig 3. The results demonstrate that this particular subline retains its sensitivity to all three anti-estrogens, although unlike the parental MCF-7 cell line appears to be sensitive to the pure anti-estrogen ZM 182,780 only at higher concentrations (> 1 μM), even though the overall rate of cell death appears to be of the same order of magnitude (approximately 40% of the cells appear to survive anti-estrogen therapy for 72h). Most of the sublines display similar sensitivities to the individual anti-estrogens although we have not yet determined whether the (minor) variations we see are reproducible or biologically significant.

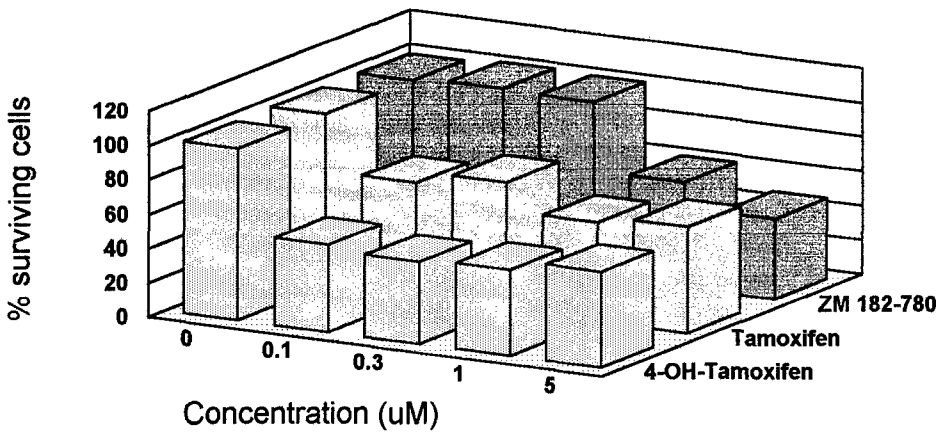



**Figure 3**: Dose response of MCF-7 $^{GFP}$ cells to ZM182,780, tamoxifen or 4-hydroxytamoxifen. MCF-7$^{GFP}$ cells were incubated with each of the indicated anti-estrogens for 72h. The cells were harvested and the cell viability was assessed using crystal violet and compared to untreated cells incubated for the same length of time. Results are average of two independent experiments.

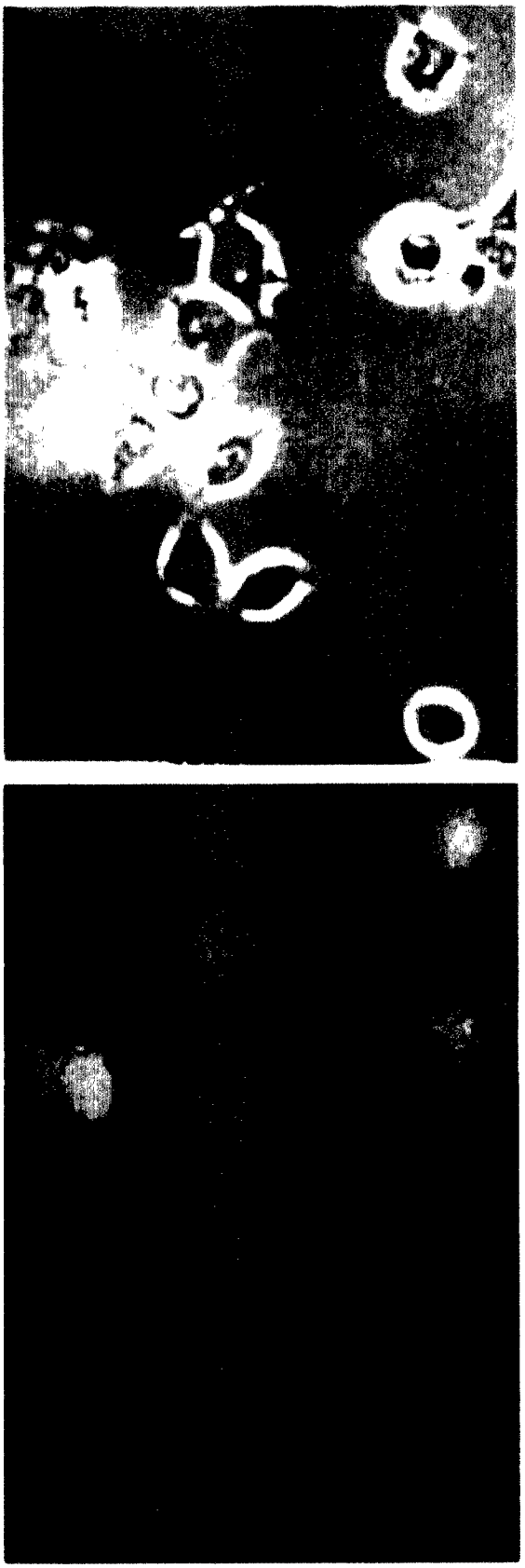
Our preliminary data, based on RT-PCR, also demonstrate that anti-estrogen (ZM 182,780) treatment of these sublines induces the synthesis of several extracellular proteases (notably cathepsin B ) while down-regulating the synthesis of at least one inhibitor of the matrix metalloproteases (TIMP-1). While these data clearly offer support to our hypothesis, they need to be repeated under rigorous experimental conditions to confirm this observation and to determine which other extracellular proteases (and acid hydrolases) are induced after treatment with anti-estrogens and which inhibitors of matrix metalloproteases and cathepsins are simultaneously down regulated.

The transfer of our laboratory from the Adirondack Biomedical Research Institute (formerly the W. Alton Jones Cell Science Center) in Lake Placid to the Department of

Biological Sciences at the University of Notre Dame has resulted in a short hiatus . However the new laboratory is now operational, and the MCF-$7^{GFP}$ cells have been resurrected and are now are being grown up to ensure the rapid continuation of the research. We do not anticipate any major problems, and we expect to complete the first two specific aims on schedule.

Our next goal is to confirm that a small population of cells that survive anti-estrogen therapy induce the proteases and acid hydrolases required for the acquisition of the invasive phenotype. We will measure the ability of the MCF-$7^{GFP}$ cells to invade through Matrigel in a modified Boyden chamber assay, by monitoring their intrinsic fluorescence as they pass through the Matrigel and membrane of the chamber before and after treatment with anti-estrogens. We will then establish whether or not other modalities that induce cell death (TNF$\alpha$ and vitamin D analogs) also induce the same phenotypic and epi-genetic changes as seen with the anti-estrogens.

We are also laying the ground work for the third specific aim, since we have initiated an *in vivo* protocol designed to demonstrate that MCF-$7^{GFP}$ cells, when grown as xenografts in the mammary fat pads of nude mice, grow at the same rate as wild type cells.



Received
10/29/2001

**DEPARTMENT OF THE ARMY**
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND AND FORT DETRICK
810 SCHRIEDER STREET, SUITE 218
FORT DETRICK, MARYLAND 21702-5000

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Oct 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for the Accession Document Numbers listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Enclosure